REGULATION OF HES1 DURING ENDOPLASMIC RETICULUM STRESS

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

As the entry point for the secretory pathway, the endoplasmic reticulum (ER) is responsible for folding and processing secreted proteins. ER stress occurs when the folding capacity of the ER is exceeded by incoming proteins, thus resulting in an accumulation of potentially harmful misfolded proteins. This condition activates the unfolded protein response (UPR). The UPR consists of three independent signaling branches that alleviate ER stress by altering gene expression to decrease the incoming protein load while simultaneously increasing the folding capacity of the ER. During chemically induced ER stress, there is strong increase in the abundance of the mRNA coding for the mammalian transcription factor hairy and enhancer of split 1 (Hes1). Since Hes1 plays critical roles in development and cancer, the goal of this thesis was to determine the mechanism behind the increase in Hes1 mRNA, discover the protein's downstream targets during ER stress, and determine the overall consequences for the cell.

Here we show that Hes1 greatly increases cellular viability during conditions of ER stress. We also show that regulation of Hes1 during ER stress in mammalian cells is dependent on the UPR factor Perk. Furthermore, this regulation is due to a dramatic increase in the stability of the mRNA. Previous work in *D. melanogaster* shows that Hairy represses Idh genes during hypoxia-induced ER stress. Interestingly, we find that Hes1 is responsible for suppressing the synthesis of Idh3α mRNA during ER stress. Additional work is required to determine the full consequences of this regulation of Idh3α as well as identifying other targets for Hes1.

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CHAPTER 1

INTRODUCTION

ER Stress and the UPR

The endoplasmic reticulum (ER) folds and processes proteins entering the secretory pathway. ER resident chaperones assist proteins to fold into the proper configuration. Normally, a balance is maintained between the incoming protein load on the ER and its ability to fold them correctly. Various stimuli (biochemical, hypoxia, pathological, etc.) can destabilize this balance, facilitating the accumulation of misfolded proteins, which in turn are potentially harmful to the cell. This imbalance, called ER stress, activates signaling pathways which are responsible for restoring the balance between the ER's folding capacity and the incoming protein load. These pathways are designated as the Unfolded Protein Response (UPR) (Moore and Hollien, 2012).

The UPR consists of three main signaling branches, each stemming from a distinct ER membrane-spanning protein, inositol-requiring enzyme 1 (Ire1), protein kinase RNA-like ER kinase (Perk), and activating transcription factor 6 (Atf6) (Walter and Ron, 2011) (Figure 1). All three pathways are activated by the accumulation of misfolded proteins in the ER. Ire1 oligomerizes and functions as an endoribonuclease to cleave an unconventional intron from the mRNA encoding X-box binding protein 1 (Xbp1) (Calfon *et al*., 2002; Li *et al.,* 2010). This splicing causes a frameshift in the mRNA that leads to the translation of the active Xbp1 transcription factor (Yoshida *et al*., 2001). Ire1 also degrades certain mRNAs associated with the ER in the process of regulated Ire1 dependent decay (RIDD) (Hollien and Weissman, 2006; Hollien *et al*., 2009).

Upon sensing stress, Perk dimerizes and autophosphorylates (Moore and Hollien, 2012). Perk phosphorylation has two significant effects. First, Perk phosphorylates eIF2α which inhibits its ability to be recharged with GTP and a Met-tRNA$_i$ prior to translation and thus greatly reduces translation initiation (Krishnamoorthy *et al.,* 2001). This alleviates ER stress by reducing the incoming protein folding load on the ER (Harding *et al.,* 1999). This phosphorylation of eIF2α also causes the second effect. Paradoxically,

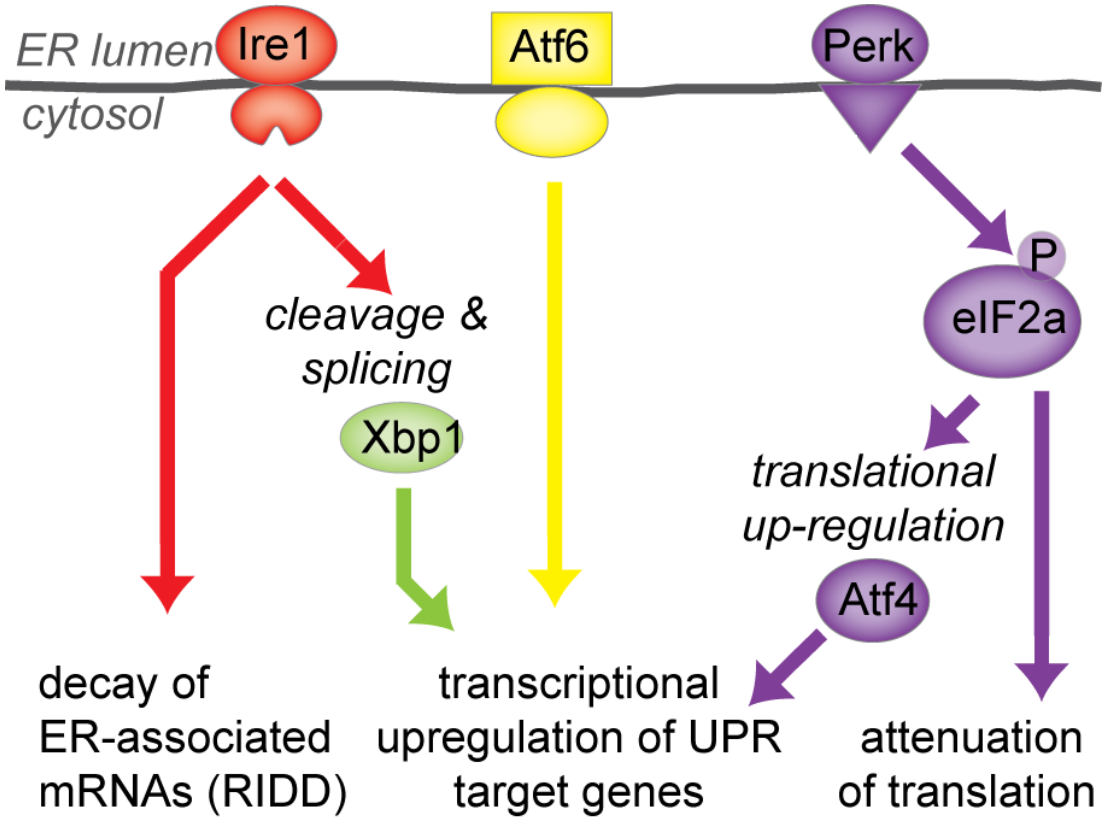

Figure 1. The Unfolded Protein Response. The UPR alleviates ER stress by decreasing the incoming protein load and increasing the folding capacity of the ER.

transcripts like Atf4 which contain uORFs are translated more efficiently during ER stress (Harding *et al.,* 2000). Atf4 then translocates to the nucleus and upregulates genes responsible for alleviating ER stress (Harding *et al.,* 2003). The third sensor, Atf6, translocates to the golgi apparatus during ER stress, where it is proteolytically cleaved to release its cytosolic domain (Haze *et al.*, 1999). The cytosolic domain is then free to translocate to the nucleus to affect transcription (Wang *et al.*, 2000).

Overall, the three branches of the UPR decrease the incoming protein load of the ER by attenuating translation and degrading specific ER-associated mRNAs while also increasing the folding capacity of the ER by upregulating genes encoding ER chaperones and other proteins involved in the secretory pathway (Shi *et al.*, 1998; Harding *et al.*, 1999; Travers *et al.*, 2000; Hollien and Weissman, 2006; Hollien *et al.*, 2009; Walter and Ron, 2011; Moore and Hollien, 2012). If the balance is unable to be restored and the cell is exposed to prolonged ER stress, then the UPR signals for cellular apoptosis (Logue *et al.*, 2013).

The effects of the UPR could have a significant impact on other signaling pathways throughout the entire cell. Upregulation of the transcription factors Xbp1, Atf4, and Atf6 has the potential to increase expression of numerous genes. In addition, the general attenuation of translation caused by Perk through the phosphorylation of eIF2α and the decay of select transcripts by RIDD affects global gene expression. The full scope of the UPR's effects remains unknown; therefore, current studies are investigating the impact of the UPR on other cellular signaling pathways.

UPR and Hes1

We have found that the mammalian transcription factor Hes1 (hairy and enhancer of split 1) is upregulated during ER stress. As a bHLH transcription factor, Hes1 binds DNA and regulates gene expression (Sasai *et al.,* 1992). Hes1 and the other members of

its family form homodimers and heterodimers with their HLH domains while the basic portions are free to interact with the DNA (Murre *et al.*, 1989). Most bHLH proteins recognize the canonical E-box binding site (CANNTG) (Blackwell and Weintraub, 1990). Although they can weakly associate with the E-box site, the Hes proteins bind preferentially to the N-box binding site (CACNAG), which could be a result of the uniquely conserved proline residue within their basic region (Sasai *et al.,* 1992).

Hes1 has been characterized as a transcriptional repressor. It accomplishes this repression through two mechanisms: active and passive. The C-terminus of Hes1 contains the four amino acid sequence Trp-Arg-Pro-Trp (WRPW). Hes1 uses this domain to associate with the corepressor protein Groucho. This complex is then able to bind to the N-box of a target gene to actively repress expression (Akazawa *et al.,* 1992; Paroush *et al.,* 1994; Fisher *et al.,* 1996). The passive repression arises from the heterodimers that bHLH factors form. Through its HLH region, Hes1 is able to heterodimerize with other bHLH factors that act as transcriptional activators. Since Hes1 preferentially associates with the N-box, this heterodimer is unable to bind to the E-box to activate transcription. Therefore, Hes1 passively suppresses gene expression by sequestering the transcriptional activator (Sasai *et al.*, 1992). Through these two methods, Hes1 suppresses differentiation within the cell by inhibiting transcription of differentiation genes even in the presence of certain signaling factors (Kageyama, 2000). This suppression of differentiation by Hes1 maintains a pool of progenitor cells that is used to achieve the various cell types required during development (Ishibashi *et al.*, 1995; Kageyama *et al.*, 2005; Fischer *et al.*, 2007).

Interestingly, Hes1 regulates its own expression. The Hes1 promoter contains several Hes1 binding sites that, when altered, reduce suppression of the Hes1 gene (Takebayashi *et al.*, 1993). Therefore, high levels of Hes1 protein result in decreased transcription of the Hes1 gene. However, the half-lives of the Hes1 protein and mRNA are both very short. Therefore, the degradation of the Hes1 protein due to its short half-life

allows transcription to initiate again at the Hes1 gene. This results in an oscillating gene expression pattern with alternating periods of high mRNA levels followed by high protein levels (Hirata *et al.,* 2002).

Notch signaling was discovered as the first regulating pathway of Hes1 expression (Jarriault *et al.*, 1995; Ohtsuka *et al.*, 1999). Since then, several Notch-independent pathways have also been documented to regulate Hes1: Hedgehog signaling, TGFalpha/RAS/MAPK, and c-Jun N Terminal kinase pathway (Curry *et al.*, 1995; Stockhausen *et al.*, 1995; Ingram *et al.*, 2008; Wall *et al.*, 2009). The upregulation of Hes1 during ER stress indicates that yet another pathway, the UPR, controls the expression of Hes1. This study will investigate the UPR mechanism controlling the upregulation of Hes1 during ER stress and the downstream consequences of this regulation.

Hes1 and Metabolism

Our main candidates for the downstream targets of Hes1 during ER stress were the genes involved in the TCA cycle. This work is based on the work by Zhou *et al.*, who generated a *Drosophila melanogaster* strain adapted to hypoxia, a known inducer of the UPR. They noticed several differences between the gene expression of the hypoxia-adapted and the control flies. Several metabolic genes such as isocitrate dehydrogenase (Idh) were significantly down-regulated. The regulatory regions of the suppressed metabolic genes contain characterized binding elements of the transcription factor Hairy, the *D. melanogaster* homologue of Hes1. Microarrays showed increased expression of Hairy, and a Hairy loss-of-function mutant restored the metabolic genes to or above normal levels. Therefore, they concluded that Hairy functioned as a metabolic switch during hypoxia.

Interestingly, work in our lab showed that *D. melanogaster* and mammalian cells exposed to ER stress also downregulate the TCA cycle genes, in a manner consistent with

their regulation by Hairy. Hairy was found responsible for the observed regulation in *D.* melanogaster cells, but the mechanism of regulation was not determined in mammalian cells. We therefore sought to determine whether key TCA cycle genes like Idh3α are regulated by Hes1 during ER stress in mammalian cells.

Hes1 and Cancer

This study has important implications for understanding the phenomenon in cancer cells known as the Warburg Effect. Normally, cells use glycolysis to create pyruvate from glucose. This step in metabolism produces a small amount of energy in the form of ATP. In order to maximize the energy harvested from glucose, the product of glycolysis pyruvate is used to create acetyl-CoA which proceeds through the TCA cycle. This process provides the reducing equivalents needed to complete oxidative phosphorylation. Oxidative phosphorylation produces the majority of the energy for the cells in the form of ATP. Up to 36 ATP can be harvested from completing the entire metabolic process (Vander Heiden *et al.*, 2009).

In hypoxic conditions, cells are unable to perform oxidative phosphorylation to produce energy. Instead, they rely on an increase in the less efficient glycolysis pathway to generate the needed energy and convert the excess pyruvate product into lactate (Semenza *et al.*, 1994; Wheaton and Chandel, 2010; Zheng, 2012). Cancerous cells undergo a similar glycolytic shift, emphasizing the initial metabolic steps of glycolysis while downregulating the TCA cycle and oxidative phosphorylation. However, cancer cells are unique since they display this shift even in the presence of sufficient oxygen levels. This has been designated as aerobic glycolysis, or the Warburg Effect (Gattenby and Gillies, 2004; Moreno-Sánchez *et al.*, 2007; Vander Heiden *et al.*, 2011; Dang, 2012). And although it has been studied extensively, the mechanism of this effect is still unknown.

It is possible that the UPR's metabolic regulation via Hes1 is responsible for this

effect. Similar to the Warburg Effect, an activated UPR is a common characteristic of tumors. The rapid growth of tumors results in insufficient angiogenesis to provide the tumor cells with all the appropriate nutrients. Therefore, these cells can experience hypoxia, glucose starvation, and amino acid deprivation, which are all inducers of ER stress and the UPR. Additionally, the rapid growth and division of cancer cells requires the constant synthesis of numerous proteins. The increased protein folding load on the ER can also overwhelm its capacity, resulting in the activation of the UPR (Ma and Hendershot, 2004; Wang and Kaufman, 2012; Vanderwynckel *et al.*, 2013).

Thus, the stress-inducing tumor environment and activation of the UPR could be responsible for the observed increased Hes1 expression in various cancer cells (Gao *et al.*, 2014; Yuan *et al.*, 2015). As previously mentioned, Hes1 expression protects cells from differentiation and senescence by blocking the expression of differentiation genes. Therefore, it has been concluded that the increased expression of Hes1 by cancer cells allows them to exhibit a relatively undifferentiated morphology and helps them maintain their proliferative potential (Sang *et al.*, 2010). Additionally, the increased Hes1 expression caused by ER stress and an activated UPR in cancer cells could cause the Warburg Effect by suppressing the TCA cycle and the downstream oxidative phosphorylation through the repression of the key regulating enzyme Idh3α.

Our Goals and Aims

Our goal for this research is to understand the purpose of Hes1 upregulation by the UPR during ER stress. The first portion of the research focuses on the upstream regulation of Hes1, including which branch of the UPR is responsible for this effect. The second portion of the study focuses on a downstream target of Hes1 discovered here, the TCA cycle gene Idh3α.

CHAPTER 2

MATERIALS AND METHODS

Cell Culture and ER Stress Induction

We cultured MC3T3-E1 (ATCC) and 3T3 (ATCC) cells in MEM alpha media (Invitrogen) and HEK293 (from A.V. Mariqc) cells in DMEM media (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics. All cell lines were cultured at 37°C with 5% CO_2. We added 2 mM DTT (Sigma) or 2 μM Tg (Sigma) to cell media for indicated times to induce ER stress. Five min before adding 2 mM DTT, we added 2 μg/mL ActD (Sigma) or 10 μg/mL Chx (Sigma) to cells to inhibit transcription or translation elongation, respectively. We added ISRIB, a gift from the Peter Walter lab (UCSF), to the cells at a concentration of 200 nM about 30 min before adding the ER stress inducer.

RNA Interference (RNAi)

For Perk and Hes1 siRNA experiments, we cultured cells in antibiotic free media. We followed RNAiMax (Invitrogen) protocols to transfect with organism specific siRNAs (Qiagen). The following siRNA reagents were used: Perk, Hes1, and Upf1. We controlled for the effects of the siRNA procedure by including simultaneous samples transfected with control (Neg) siRNA that do not target any mammalian gene (Qiagen). We incubated the cells with the transfection reagents for 48 h before treating with or without ER stress.

Plasmid Reporter Construction and Transfection

For the Idh3α promoter experiment, we amplified the first 1,000 bp upstream of the Idh3α transcription start site from mouse gDNA, adding the restriction site Mlu1 at the 5' end and Kpn1 at the 3' end with the following primers: CAG ACA ACG CGT CAG CCC AAG AGC TGT GTT TT (forward) and TGT CTG GGT ACC CGC GTC CAC TTC CCT CAC C (reverse). We subcloned the promoter upstream of the CDS of GFP using the Mlu1 and Kpn1 sites in a previously modified vector with hygromycin resistance (Moore and Hollien, 2015).

We then created a polyclonal stable cell line by transfecting 2 μg plasmid into

MC3T3-E1 cells using Lipofectamine 2000 (Invitrogen). We replaced media after 1.5-2 h, and allowed cells to recover for an additional 24-36 h before passaging and adding 100 μg/mL hygromycin B. We continually selected for hygromycin B resistant cells over a 2-3 week period and cultured in 100 μg/mL hygromycin B thereafter.

RNA Preparation and Analysis

We isolated mRNA from cultured cells using the Zymo Research Quick-RNA MiniPrep Kit or using the Trizol reagent (Invitrogen). We then synthesized cDNA using 1-2 μg of total mRNA as a template, a T18 primer, and a MMLV reverse transcriptase (NEB). We used qPCR to measure the relative mRNA levels using the Mastercycler ep realplex (Eppendorf) with SYBR green as the fluorescent dye. We measured each sample in triplicate, and mRNA levels were normalized to those of ribosomal protein 19 (Rpl19) mRNA. Primer sequences were as follows: Rpl19 (mouse), CTG ATC AAG GAT GGG CTG AT (forward), GCC GCT ATG TAC AGA CAC GA (reverse); Rpl19 (human), ATG TAT CAC AGC CTG TAC CTG (forward), TTC TTG GTC TCT TCC TCC TTG (reverse); Hes1 (mouse), TAA CGC AGT GTC ACC TTC CA (forward), AGG CGC AAT CCA ATA TGA AC (reverse); Hes1 (human), CTG TCA TCC CCG TCT ACA CC (forward), AGG CGC AAT CCA ATA TGA AC (reverse); Idh3α, GAC TTA ATT GCC GAG GTG GA (forward), CCC AGA CAG GGA CAT CTG TT (reverse); Upf1, CCA AGC AGC TAA TCC TCG TC (forward), GCA ATG AGC CCT CGT AGA AG (reverse).

Polyribosome Fractionation

We cultured Hek293 cells in two 75 mL flasks. The control flask remained unstressed. We stressed the second flask with 2 mM DTT for 1 h in DMEM media. We then aspirated the media, replaced it with DMEM media (100 μg/mL CHX) or with DMEM media (2 mM DTT and 100 μg/mL CHX), respectively, and left the flasks at room temperature for 5 min. To resuspend the cells, we aspirated the media and added trypsin

with or without 2 mM DTT to the flasks. We then pelleted the cells by centrifugation for 5 min at 800xg. We used 5 mL PBS with 100 μg/mL CHX to wash the samples. We transferred 0.75 mL of each sample to a 1.5 mL eppendorf tube, spun it for 5 min at 1000xg, resuspended the cells in 250 μL PBS and 750 μL Trizol LS, and stored the samples at -70°C. We centrifuged the remaining 4.25 mL of each sample for 5 min at 1,000xg. We aspirated the supernatant and resuspended the cells on ice for 3 min in low salt buffer (LSB – 20 mM TrisBase, 10 mM NaCl, 3 mM MgCl, Ph 7.4) containing 100 μg/mL CHX. We added 125 μL of LSB lysis (20 mM TrisBase, 10 mM NaCl, 3 mM MgCl, 200 mM sucrose, 1.2% triton X-100, pH 7.4) to each sample and centrifuged them for 1 min at 4°C at 20,000xg. We transferred 450 μL of the supernatant from each sample to a new 1.5 Ml eppendorf tube containing 15 μL 5 M NaCl and 50 μL LSB. We then loaded each sample onto a premade sucrose gradient in a Beckman Polyallomer Ultracentrifuge tubes 13x51 mm which were then placed in a SW55Ti rotor. We created the ribosomal gradient by centrifugation for 60 min at 47,000 rpm at 4°C with no brakes. To separate and collect the ribosomal fractions from the sucrose gradients, we used an ISCO UA-6 Absorbance Detector. We collected the initial 1 mL of the each sample for the monosomal fractions and subsequent samples of 250 μL for the polysomal fractions. Following Trizol purification of mRNA, 3 μL of each sample was used for cDNA synthesis for qPCR analysis.

Cell Viability

We passaged 0.25 mL of MC3T3 cells into 1.75 mL of DMEM antibiotic free media. The following day, we performed RNAi using control and Hes1 siRNA. After allowing the RNAi 48 h to properly take effect, we induced ER stress by adding different concentrations of DTT or Tg for 4 or 7 h, respectively. We then aspirated the media and added 250 μL of trypsin to resuspend the cells. We added 250 μL of media to neutralize the trypsin and transferred each sample into a fresh 1.5 mL eppendorf tube. We counted the cells by

adding 10 μL of cells onto a hemocytometer and visualizing under a light microscope. We normalized all results to the unstressed control RNAi sample.

Western Blot

We washed cells in PBS before lysing in 1x RIPA buffer (25 mM Tris, Ph 7.6, 150 mM NaCl, 1% NP-40, 1% Na- deoxycholate, and 0.1% SDS) with protease inhibitors (Thermo scientific). We determined protein concentrations using a BCA assay (Thermo scientific). We resolved about 20 μg of protein on NuPage Bis-Tris 4–12% gels (Invitrogen), transferred them to nitrocellulose membranes, and probed for total Idh3α (Abcam ab58641, 1:500) or Hes1 (Santa Cruz sc-25392, 1:1000) followed by a secondary IRDye 800CW antibody (Licor 926-32210, 1:10000). We visualized immunoblots using a Licor Odyssey imager. We used the Novex Sharp Pre-stained protein ladder for size comparison.

CHAPTER 3

RESULTS

Hes1 mRNA Upregulation

Previous work had shown that Hairy (in S2 cells) and Hes1 (in mouse fibroblasts) were induced by ER stress. We first tested whether this induction occurs in response to different stress inducers (2 mM DTT (dithiothreitol) and 2 μM Tg (thapsigargin)) in two different cell lines. DTT induces ER stress by reducing the disulfide bonds that stabilize the tertiary structure of proteins. Tg inhibits the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), thereby depleting the calcium stores within the ER (Oslowski and Urano, 2013). As seen in Figure 2, Hes1 mRNA levels increased under the influence of both stress inducers, in both human HEK293 cells and in the mouse osteoblast cell line MC3T3. This regulation is also seen in Min6 cells after Tg treatment (Szabat *et al.*, 2011). Therefore, we conclude that Hes1 mRNA levels increase during ER stress in a variety of mammalian cell types.

Hes1 and Cell Viability During ER Stress

Since Hes1 mRNA levels increased with DTT and Tg, we speculated that its corresponding protein is important for the cellular response to ER stress. We measured the viability of cells during ER stress while simultaneously silencing Hes1. We performed a Hes1 RNAi treatment in MC3T3 cells to prevent Hes1 from performing its normal function during ER stress. Following 48 h of siRNA treatment, we treated the cells for 4 h with concentrations of DTT ranging from 0.25 mM to 4 mM. We performed a similar experiment stressing cells for 7 h with Tg with concentrations ranging from 0.5 μM to 4 μM. We then collected the cells and counted the number of cells at each concentration. A decrease in the cell count in comparison to the control sample would indicate a reduction of cellular viability resulting from either the Hes1 RNAi treatment or the induction of ER stress. We then normalized each concentration cell count to the unstressed RNAi control cells.

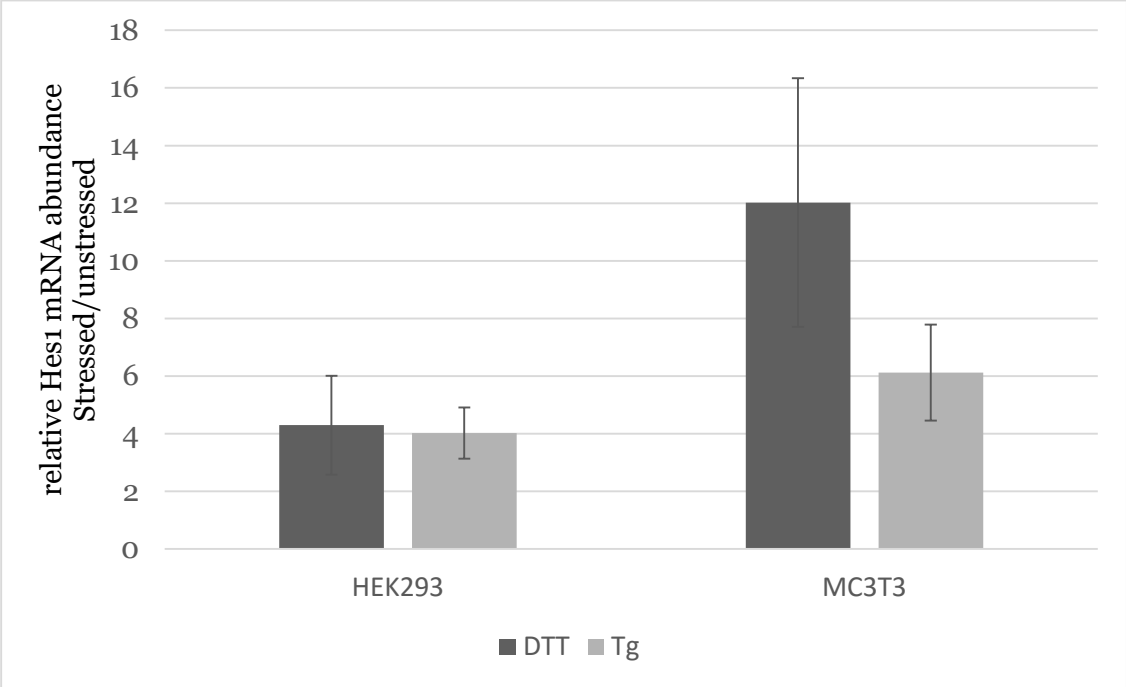

Figure 2. Hes1 mRNA levels increase during ER stress. HEK293 cells were stressed with 2 mM DTT and 2 μM Tg for 3 and 2 h, respectively. MC3T3 cells were stressed with 2 mM DTT for 5 h and with 2 μM Tg for 2 h. The mRNA was purified and used to make cDNA for qPCR. Hes1 mRNA levels were normalized to Rpl19 mRNA levels. Data are average of three independent experiments +/- standard deviation.

As seen in Figure 3, induction of ER stress with the increasing concentrations of DTT gradually reduced the viability of the control cells until they reached 80% at 4 mM DTT and 70% at 4 μM Tg. While Hes1 RNAi treatment alone negligibly reduced the viability of the MC3T3 cells, Hes1 silencing combined with increasing concentrations of DTT greatly reduced cell viability, with the biggest effect seen at the highest concentrations. Cellular viability was reduced by nearly half with Hes1 RNAi with 4 mM DTT. These findings were supported by the Tg experiment which showed a one-third reduction in viability at the highest concentrations of Tg. Overall, this indicates that Hes1 plays a significant role in the cellular response to ER stress.

Hes1 mRNA Regulation by Perk

Knowing that Hes1 was important for the cellular response to ER stress, we then determined which branch of the UPR was responsible for its regulation. Previous work with S2 cells indicated that the Hes1 homologue Hairy is regulated by Perk (unpublished data). To test if this is also true in mammalian cells, we inhibited the Perk branch of the UPR in MC3T3 and HEK293 cells prior to induction of ER stress and determined the effects on the regulation of Hes1 mRNA. We used two techniques for blocking the Perk pathway, Perk RNAi and ISRIB treatment. ISRIB is a small compound that inhibits the signaling through the Perk pathway by rescuing the effects of eIF2α phosphorylation and restoring general translation to the cell (Sidrauski *et al.*, 2013; Sidrauski *et al.*, 2015).

As seen in Figure 4.A, the negative control RNAi did not prevent the increased levels of Hes1 mRNA during ER stress induced with 2 mM DTT. However, as seen with Hairy, Perk knockdown suppressed the upregulation of Hes1 during stress within both cell types. The ISRIB experiments confirmed these findings, shown in Figure 4.B. Hes1 mRNA levels did not significantly alter with ISRIB treatment alone. ISRIB did suppress the aforementioned upregulation of Hes1 mRNA levels during ER stress with either DTT

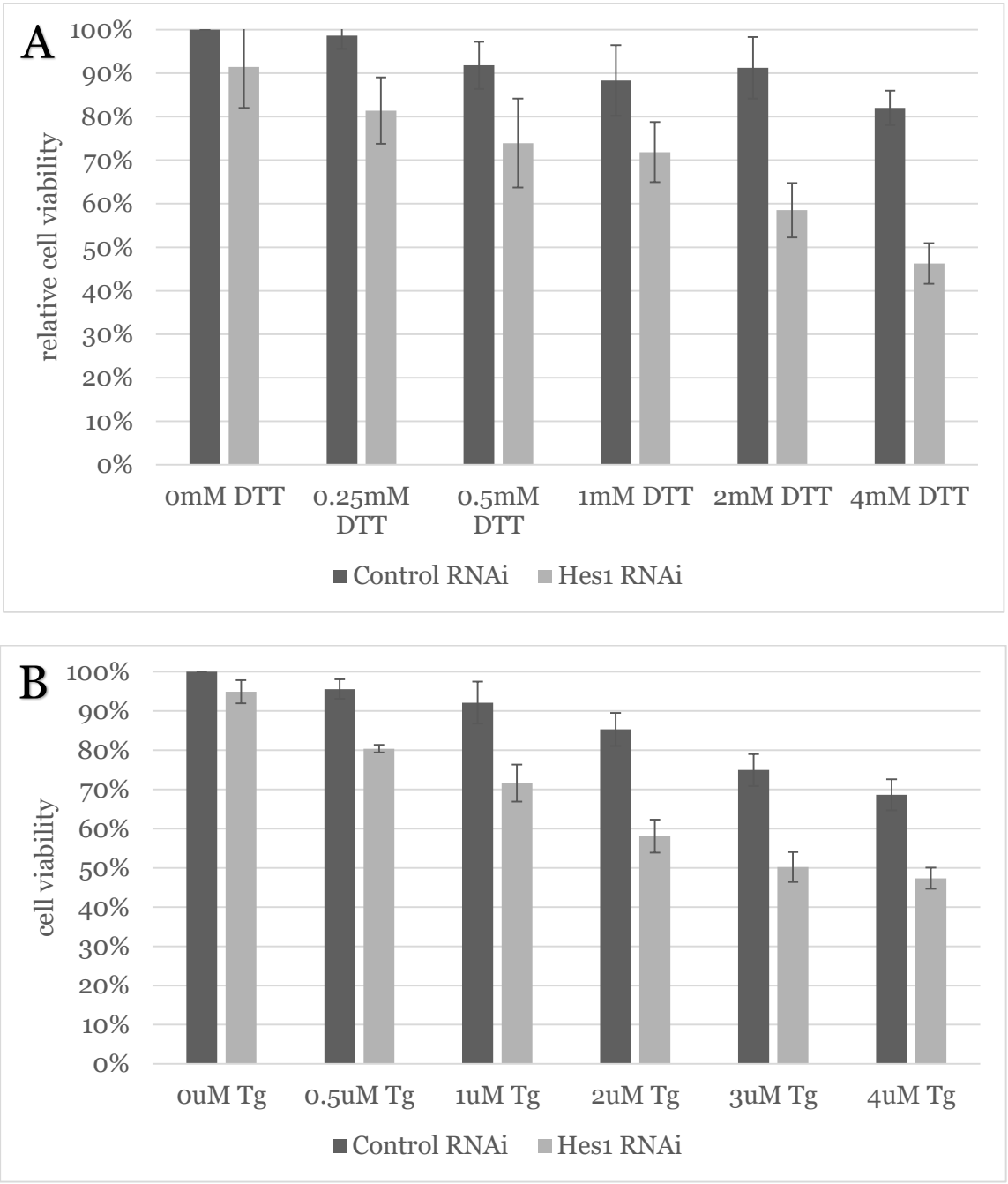

Figure 3. Hes1 RNAi decreases cellular viability during ER stress. Hes1 RNAi treatment was performed on MC3T3 cells for 48 h prior to the induction of ER stress with the indicated concentrations of DTT for 4 h (A) or Tg for 7 h (B). Cells were then counted and normalized to the unstressed control sample. Data are average of three independent experiments +/- standard deviation.

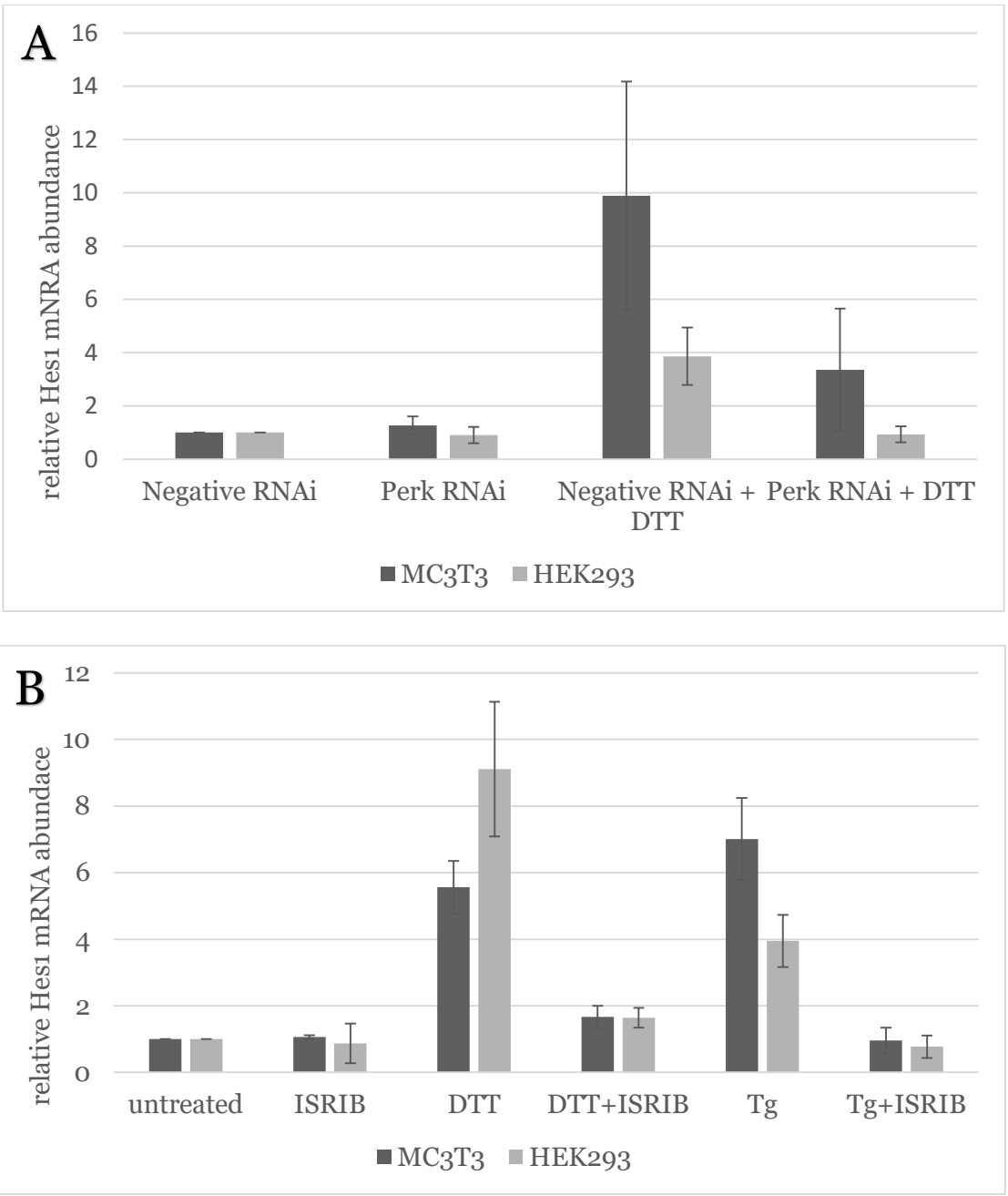

Figure 4. Hes1 mRNA regulation during ER stress is controlled by the Perk pathway. (A) Perk RNAi treatment was performed on MC3T3 and HEK293 cells for 48 h prior to induction of ER stress with 2 mM DTT for 4 h. MC3T3 data are average of 3 experiments. HEK data are average of 2 experiments. (B) MC3T3 cells were treated with 200 nM ISRIB for 30 min prior to inducing stress with either 2 mM DTT for 4 h or 2 μM Tg for 2 h. HEK293 cells were similarly treated with 200 nM ISRIB for 30 min prior to inducing stress with either 2 mM DTT for 2 h or 2 μM Tg for 2 h. HEK293 DTT data are average of 3 experiments. HEK293 Tg data are average of 2 experiments. MC3T3 DTT data are average of 2 experiments. MC3T3 Tg data are average of 4 experiments. mRNA was purified and used to synthesize cDNA for qPCR analysis. Normalized to Rpl19. +/- standard deviation.

or Tg. Therefore, we concluded that regulation of Hes1 during ER stress is controlled by the Perk signaling branch.

Hes1 mRNA Stability

We then investigated the mechanism that Perk employs to regulate Hes1 expression. We first tested whether Hes1 upregulation is due to an increase in transcription or a decrease in its degradation. We treated HEK293 cells with actinomycin D to inhibit new transcription of Hes1 mRNA and measured the rate of decay of the present Hes1 mRNA levels with and without induction of ER stress. Hes1 mRNA levels were reduced to below 50% following only 1 h of inhibition of new transcription and ultimately reached roughly 10% of initial levels after 4 h (Figure 5). Inducing ER stress immediately following inhibition of transcription significantly decreased the degradation of the mRNA throughout the times measured. Interestingly, the difference of mRNA levels between the stressed and unstressed samples at 2 and 3 h is equivalent to the upregulation of Hes1 mRNA in HEK293 cells during ER stress in Figure 1. This suggests that the majority if not entirety of upregulation of Hes1 mRNA levels during ER stress is a product of its increased mRNA stability due to decreased degradation.

Polysome Analysis of Hes1

We know that Hes1 is important for viability during ER stress (Figure 3). As it is highly unlikely that the mRNA itself is responsible for this effect, we turned our attention towards the protein levels of Hes1 during ER stress. We speculated that the increased stability of the Hes1 mRNA allowed for more translation and therefore higher levels of Hes1 protein during ER stress.

Unfortunately, we were unable to detect the Hes1 protein under any condition through Western blot. Since a direct approach failed, we decided to measure protein levels indirectly by measuring Hes1 mRNA translation with or without ER stress via polysome

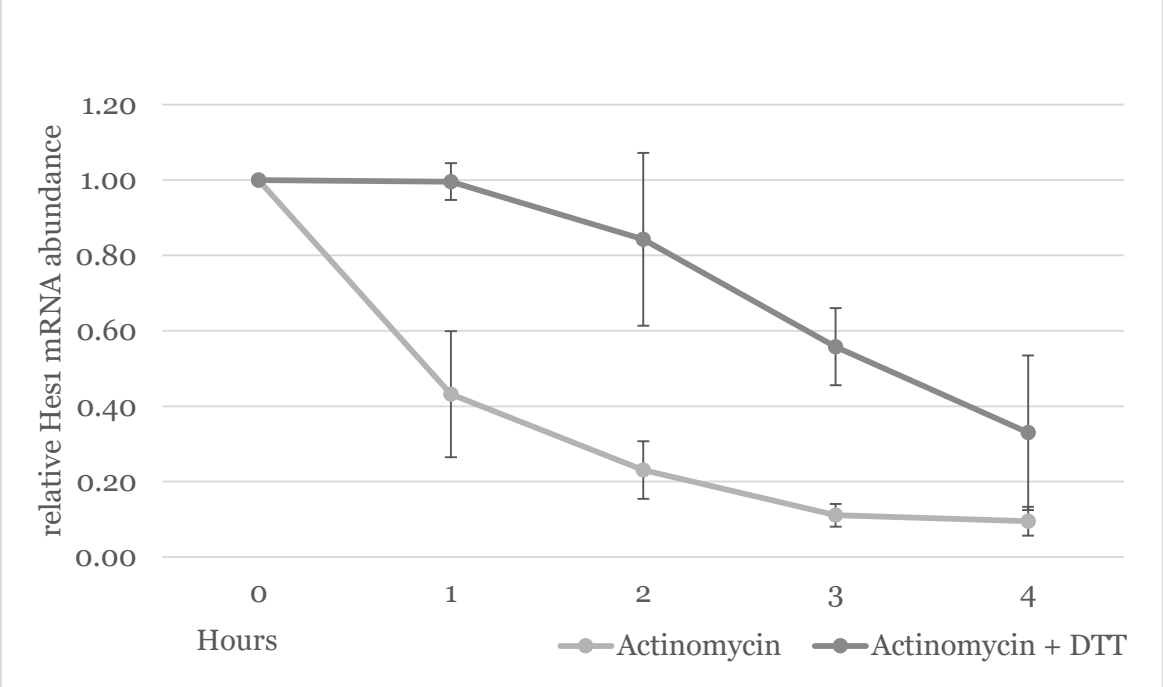

Figure 5. Hes1 mRNA stability is increased during ER stress. HEK293 cells were treated with 2 µg/mL of actinomycin D. For the stressed samples, 2 mM DTT was added 5 min after the addition of actinomycin D At the indicated times, mRNA was isolated for analysis by qPCR. Normalized to Rpl19. Data are the average of three independent experiments +/- standard deviation.

analysis.

As mentioned previously, ER stress causes the transmembrane protein Perk to dimerize and autophosphorylate. Perk then causes a global attenuation of translation by phosphorylating eIF2α, a component of the preinitiation complex. Paradoxically, transcripts like Atf4 that contain uORFs are translationally upregulated.

Therefore, the polysome analysis of Hes1 mRNA had three possible outcomes. First, the Hes1 mRNA could increase in translation during ER stress. This effect combined with the increased mRNA abundance would result in a large increase in the Hes1 protein levels. Second, it was also possible that the Hes1 mRNA followed the general trend of mRNAs during ER stress and exhibited decreased translation. If the levels of translation were only slightly or moderately reduced, then the increased abundance and stability of the mRNA could still result in an increase in Hes1 protein levels. Third, the reduced translation of the Hes1 mRNA could have a greater effect than the increased mRNA stability, resulting in an overall downregulation of Hes1 protein levels.

Polysome analysis in HEK293 cells revealed that ER stress caused a moderate reduction of translation of the Hes1 mRNA (Figure 6). We observed that under normal conditions, the Hes1 mRNA was present only in the higher polyribosome fractions. There was no measurable product produced within the monosome or the first five fractions. In comparison, during ER stress, the Hes1 mRNA is more broadly distributed throughout the fractions, being seen in both in the monosome and the highest polysome fraction with the most product present within the middle samples. This indicated that there are fewer ribosomes simultaneously translating each Hes1 transcript during ER stress.

Although the translation of the Hes1 transcript decreased during ER stress, the increase in mRNA levels could compensate and overcome the decreased translation to achieve an overall increase in Hes1 protein levels. HEK293 cells exhibit about a four-fold increase in mRNA abundance during ER stress. The polysome analysis suggests a two-

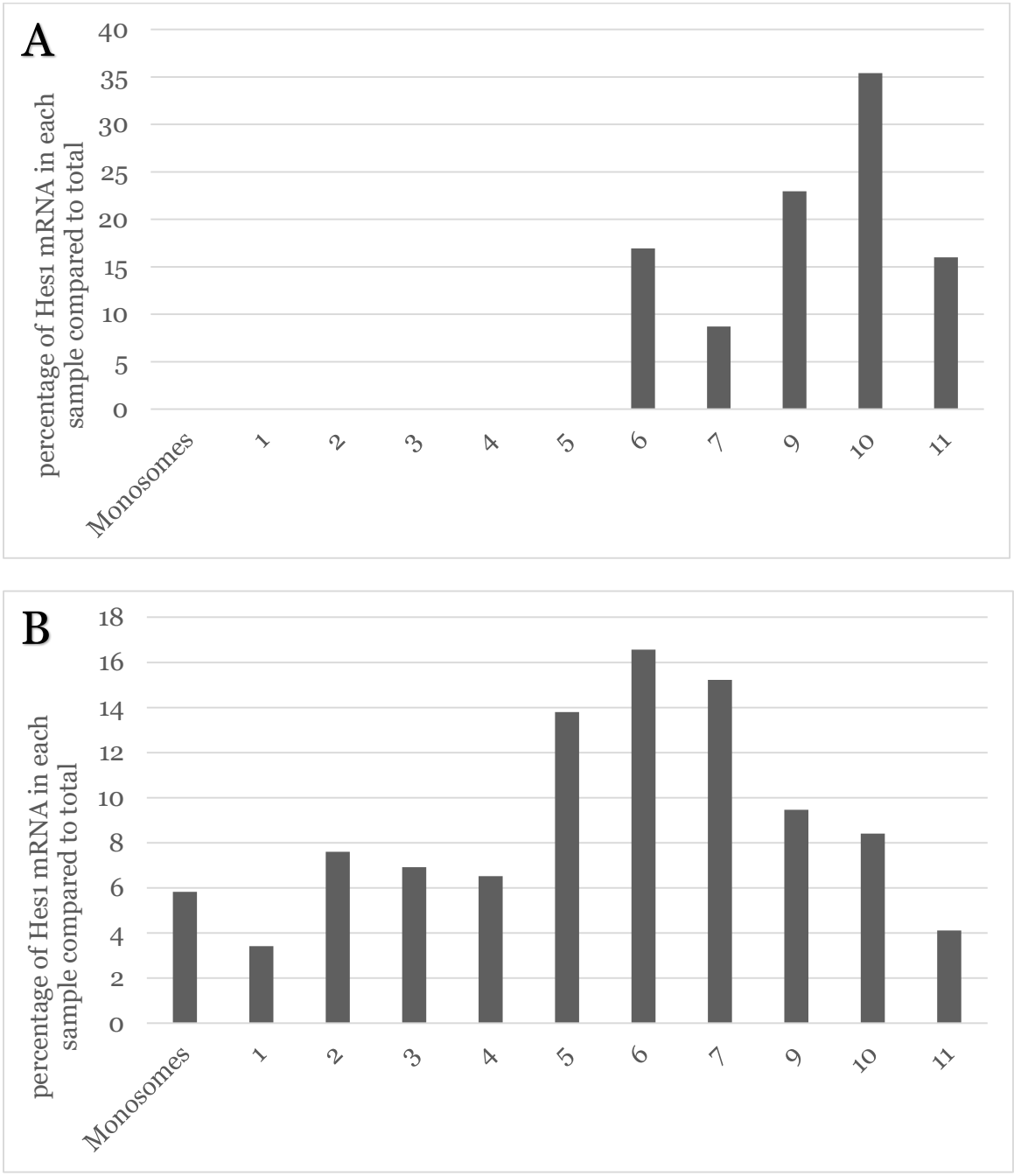

Figure 6. Fewer ribosomes are present on each Hes1 mRNA during ER stress. HEK293 cells were stressed with 2 mM DTT prior to polysome fractionation. Following fractionation, 3 µL of mRNA from each sample/fraction was used for cDNA synthesis and qPCR analysis. Sample 8 was omitted. The total amount of Hes1 product in each condition was calculated, and each fraction represents the percentage of the total Hes1 made in each individual sample in the respective condition. Data are one experiment. (A) Unstressed HEK293 cells (B) HEK293 cells stressed with 2 mM DTT.

fold reduction in translation of each transcript. Combined, the upregulation of the mRNA could overcome the reduction in translation, resulting in an overall increase in Hes1 protein levels. This experiment was conducted once, and replicates should be performed to support this finding.

Hes1 and NMD

Interestingly, the polysome analysis also provided a possible mechanism for the observed stabilization of the Hes1 mRNA during ER stress: non-sense mediated decay (NMD). NMD is a translational-dependent decay mechanism comprised of the Upf and Smg protein families. It was originally discovered to protect the cell from C-terminally truncated proteins by degrading mRNAs with a premature termination codon (PTC). Further analysis demonstrated that NMD also controls the regulation of 5-10% of normal physiologically relevant transcripts (Rehwinkel *et al.*, 2006; Brogna and Wen, 2009). Since we observed reduced degradation and translation of the Hes1 mRNA during ER stress (Figures 5 and 6), we hypothesized that the Hes1 transcript is a target of NMD. The reduced translation of the Hes1 mRNA caused by a phosphorylated eIF2α would diminish the degradation effects of the NMD pathway, thereby increasing the stability of the Hes1 mRNA.

If our hypothesis were true, Hes1 mRNA degradation would decrease with inhibition of the NMD pathway. For this purpose, we used siRNA to knockdown the core component of the NMD pathway, Upf1. A Upf1 knockdown pilot experiment within MC3T3 cells resulted in significant cell death; therefore, this experiment was performed with the similar 3T3 cell line. Following 48 h of RNAi treatment, we treated the cells with actinomycin D for 1 h to inhibit new transcription. We then measured the degradation of Hes1 alongside a known NMD target Gadd45b by purifying the mRNA for qPCR analysis. If NMD degraded Hes1, we expected that the Upf1 knockdown samples with or without

actinomycin D treatment would exhibit similar Hes1 mRNA levels to the control.

As expected, Upf1 knockdown greatly increased Gadd45b levels, confirming that it is an NMD target, and the Upf1 silencing in combination with the inhibition of transcription returned the Gadd45b levels slightly below the control (Figure 7). Conversely, Upf1 treatment failed to increase Hes1 mRNA abundance. Also, the rate of degradation of the Hes1 transcript regardless of siRNA treatment appeared similar to the findings in Figure 4. Therefore, we concluded that another mechanism besides the NMD pathway is responsible for the degradation of the Hes1 mRNA.

Hes1 Regulation of IDH3a

Considering that Hes1 increases the viability of cells even after only 4 h of induced stress (Figure 3), we wanted to determine its downstream targets. Again, previous work with its *D. melanogaster* homologue Hairy provided a candidate: Idh3α (isocitrate dehydrogenase 3α). Hairy suppressed the expression of Idh in *D. melanogaster* adapted to hypoxia (Zhou *et al.*,2008). And a loss-of-function Hairy mutant reduced the survivability of the flies in hypoxia similar to the reduction in cell viability during Hes1 silencing. Therefore, we hypothesized that the mammalian Hes1 also suppressed Idh3α during ER stress.

To test our hypothesis, we performed a Hes1 RNAi treatment for 48 h to deplete cellular Hes1 protein levels. We induced stress with 2 mM DTT for 6 h and measured the levels of Idh3α mRNA in the different conditions. We compared the changes between the stressed and unstressed samples in each RNAi treatment (Figure 8). In the control group, Idh3α mRNA levels were reduced to roughly 40% after 6 h of stress. However, this reduction was almost abolished when Hes1 was knocked-down. This suggests that Idh3α expression is repressed by Hes1 during ER stress.

We then sought to determine if the protein levels of Idh3α mirrored the changes in

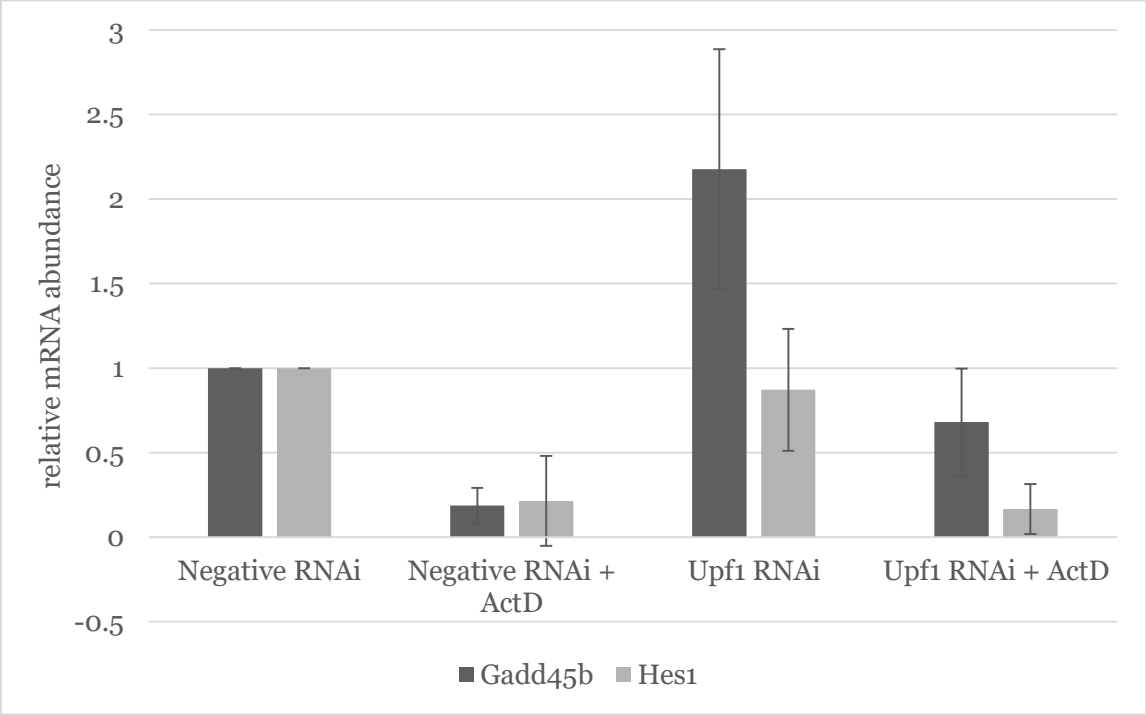

Figure 7. NMD does not degrade Hes1 mRNA. Upf1 or control siRNA treatment was performed on 3T3 cells for 48 h, following which 2 μg/mL of actinomycin D was added for 1 h. mRNA was purified and used to synthesize cDNA for qPCR analysis. Data normalized to Rpl19. Data are average of 3 experiments +/- standard deviation.

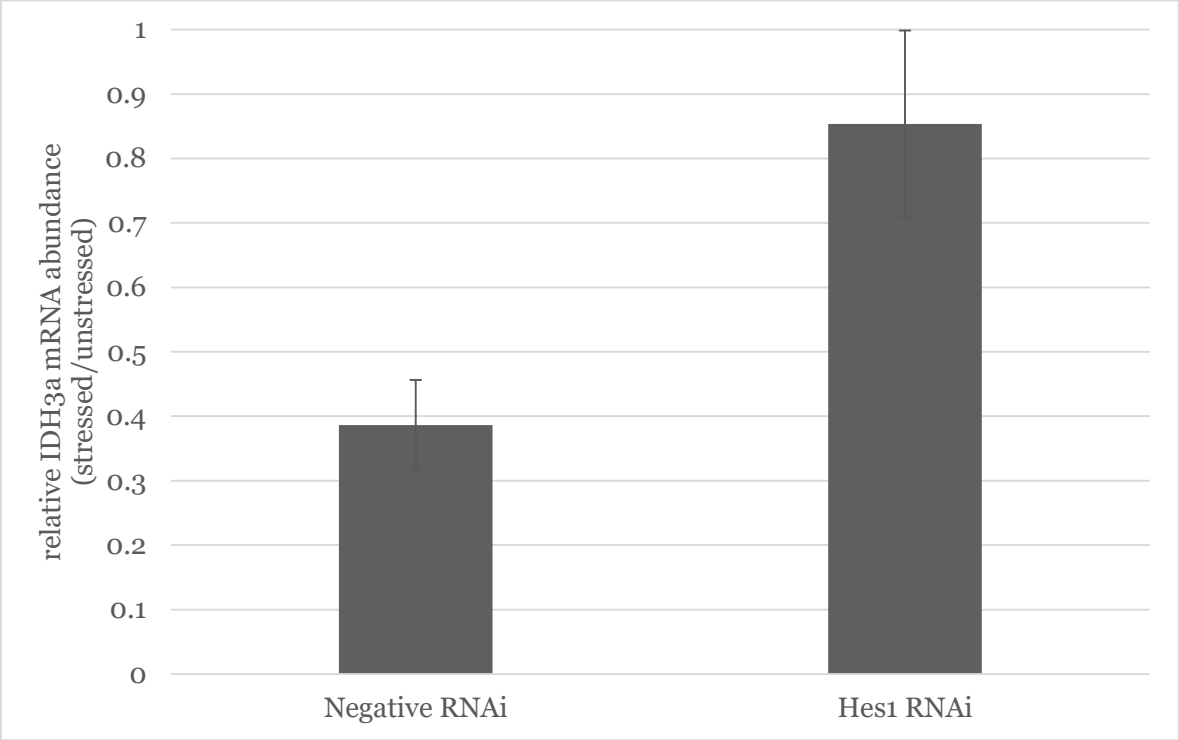

Figure 8. Idh3α mRNA is suppressed by Hes1 during ER stress. MC3T3 cells were subjected to control or Hes1 siRNA for 48 h. The cells were then stressed with 2 mM DTT for 6 h, after which the mRNA was purified and used to synthesize cDNA for qPCR analysis. The samples were normalized to Rpl19. The percent change between the stressed and unstressed samples was measured. Data are average of 2 independent experiments.

mRNA abundance during ER stress. We silenced Hes1 expression within MC3T3 cells for 48 h prior to inducing stress for 6 h with 2 mM DTT. Visualization by western blot revealed that Idh3α protein levels (~37 kDa) remained consistent regardless of silencing or ER stress (Figure 9).

Preliminary Idh3a Promoter

We then wanted to characterize the mechanism by which Hes1 represses the expression of Idh3α. Hes1 binds to the strongly to the N-box and weakly to E-box sequences within gene promoters. We examined the first 1,000 bp upstream of the Idh3α gene for possible Hes1 binding sites. As seen in Figure 10.A, the tentative promoter contained several candidate binding sites. The preferential N-box target binding sequence (CACNAG) is located 36 bp and 898 bp upstream of the transcription start site. Three E-box sequences (CANNTG) are located 165 bp, 224 bp, and 857 bp upstream. We hypothesized that during ER stress Hes1 binds to one of these sites and represses transcription of the Idh3α gene. Therefore, we constructed a reporter that contains 1000 nucleotides of the Idh3α promoter followed by GFP. A reduction in GFP mRNA levels during ER stress would indicate that the promoter was sufficient to report regulation by the UPR.

After the creation of a stable MC3T3 cell expressing our GFP reporter, we induced ER stress with 2 mM DTT for 4 and 7 h and collected mRNA for analysis. The GFP mRNA levels were not changed during ER stress (Figure 10.B). It could be that our reporter construct is insufficient to represent the regulation of Hes1 on the selected portion of the Idh3α promoter. Possible Hairy binding sites for the *D. melanogaster* homologue Idh are up to 5 kb upstream from the start site. Therefore, we could construct future GFP reporter genes controlled by a longer Idh3α promoter sequence to test their regulation by Hes1.

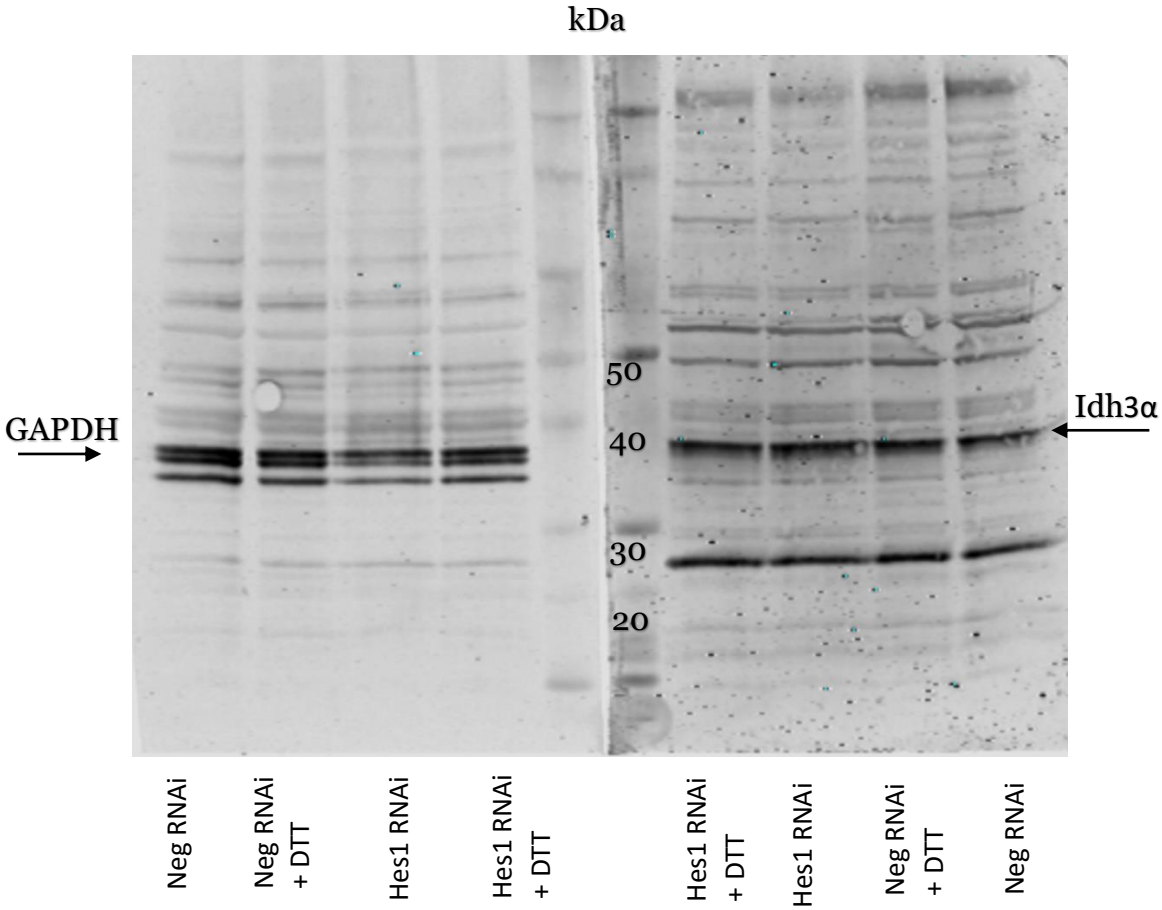

Figure 9. Idh3α protein levels are unchanged by ER stress or Hes1 silencing. Hes1 RNAi was performed on MC3T3 cells for 48 h. They were then stressed with 2 mM DTT for 6 h. Lanes 1-5 were incubated with GAPDH antibody as a relative comparison of protein loaded within each sample. Lanes 6-10 were incubated with Idh3α antibody. Novex sharp pre-stained protein ladder was used to determine protein size. Representative of two experiments.

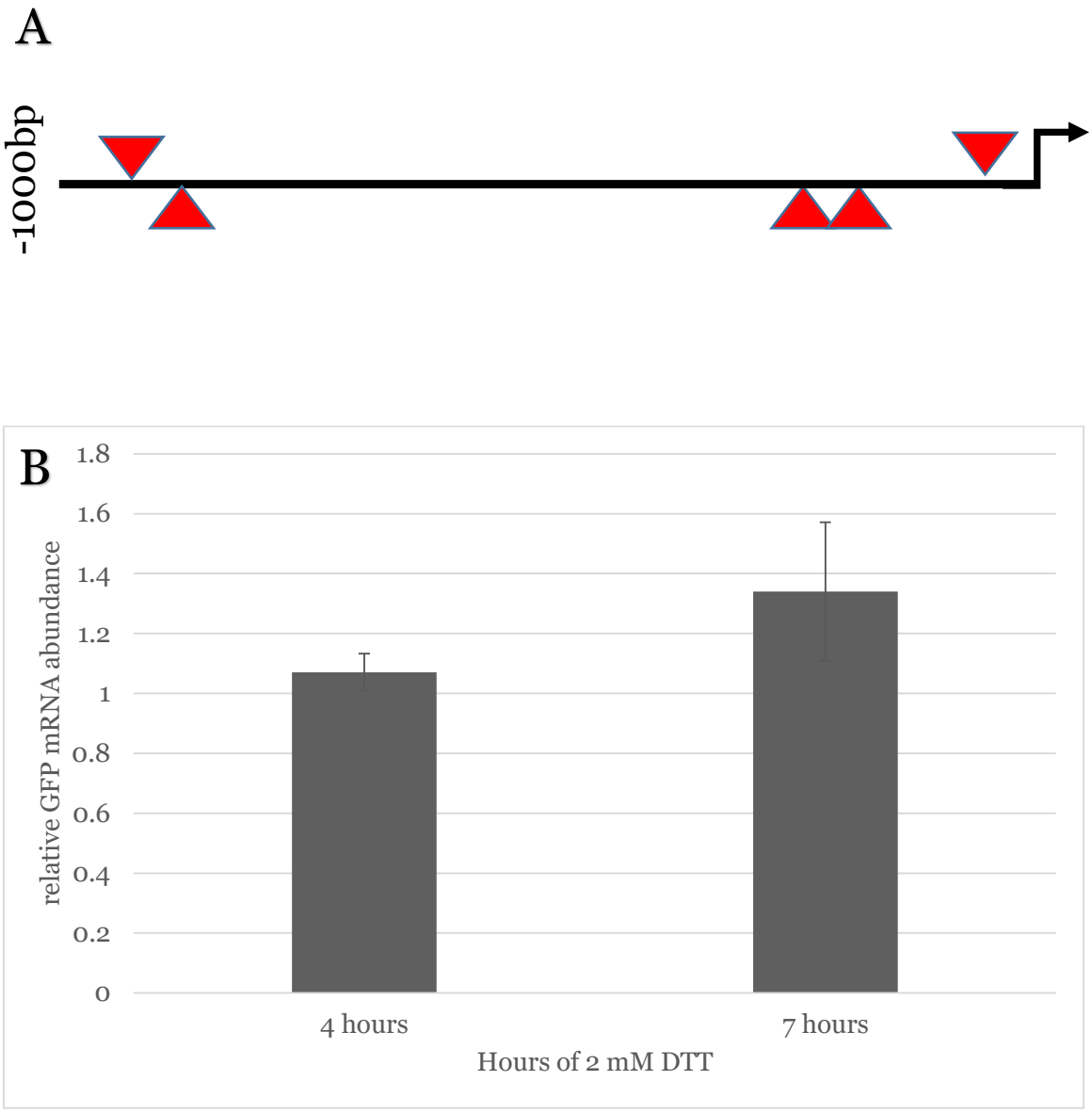

Figure 10. Hes1 does not regulate the expression of the Idh3α promoter. (A) A representation of the possible Hes1 binding sites within the 1,000 bp upstream of the Idh3α gene transcription start site. The triangles above the line represent N-box sites while the triangles below represent E-box sites. (B) MC3T3 cells expressing the GFP reporter gene were stressed with 2 mM DTT for 4 and 7 h, following which mRNA was purified for qPCR analysis.

CHAPTER 4

DISCUSSION

We have shown that chemical induction of ER stress and activation of the UPR within HEK293 and MC3T3 cells upregulates the mRNA of the transcriptional repressor Hes1 (Figure 2). We also observed that Hes1 expression is important for cell viability during ER stress (Figure 3). These data suggest that the upregulation of Hes1 is an important aspect of the cellular response to ER stress.

We also observed that the Perk signaling branch of the UPR is responsible for regulating Hes1 (Figure 3) and that Hes1 mRNA stability greatly increases during ER stress (Figure 4). The polysome analysis suggested that the decreased degradation of the Hes1 transcript is connected with its decreased translation. However, this was not due to the classic translation-dependent decay pathway NMD (Figure 7).

It is possible that the stabilization of the Hes1 mRNA is controlled indirectly during ER stress. The UPR and Perk specifically could upregulate a stabilizing protein of the Hes1 mRNA or downregulate a protein that signals for the degradation of the Hes1 transcript. Additionally, various micro RNAs are known to interact and silence Hes1 (Garzia *et al.*, 2009; Chen *et al.*, 2012). Micro RNAs (miRNA) bind to complementary sequences within mRNAs silencing their expression (Ambros, 2004). One or several of the miRNAs known to interact with the Hes1 mRNA could be downregulated by the Perk signaling branch. This would reduce the degradation of the Hes1 transcript during ER stress. Further investigations into the abundance of specific regulatory miRNAs or possible interacting protein partners during ER stress could elucidate the mechanism of Hes1 mRNA degradation.

We also discovered that Hes1 regulates the abundance of the Idh3α mRNA during ER stress (Figure 8). However, we were unable to demonstrate a similar reduction in Idh3α protein levels during ER stress (Figure 9). The inability to detect a change in protein levels could be a result of the set variables of the experiment. An exposure longer than 6 h to ER stress could be required to measure a change in Idh3α protein levels.

However, the regulation of the Idh3α mRNA through the transcriptional repressor Hes1 provides a mechanism by which the UPR may regulate metabolism during times of ER stress. The suppression of Idh3α by Hes1 would inhibit progression through the TCA cycle and oxidative phosphorylation. The levels of glycolysis could increase within a cell to compensate for the loss ATP derived from mitochondrial respiration. Previous studies confirm the regulation of metabolism by the UPR during ER stress. The overexpression of Perk and the induction of ER stress in mammalian cell cultures result in the repression of mitochondrial respiration (Muñoz *et al*., 2013; Win *et al*. 2013). Additionally, in *D*. melanogaster, the transcription factor Atf4 upregulates glycolytic genes while Hairy downregulates genes involved in the TCA cycle and oxidative phosphorylation (Lee *et al*. 2015).

The regulation of metabolism by the UPR could provide several benefits for the cell during ER stress. An output of the UPR alleviates stress by expanding the ER which requires the synthesis of cellular membrane components like glycolipids. Glycolytic intermediates are required for the synthesis of glycolipids; therefore, a downregulation of mitochondrial respiration and a subsequent upregulation of glycolysis could provide sufficient quantity of building blocks for glycolipid synthesis (Sriburi, 2004; Sriburi *et al*., 2006; Lee *et al*., 2008).

Another possible benefit for the downregulation of the TCA cycle and oxidative phosphorylation is the reduction of the formation of ROS (reactive oxygen species). ROS accumulation is damaging to the cell and signals for apoptosis. The two main sources of ROS within the cell are protein folding and mitochondrial respiration (Solaini *et al*., 2010). The formation of disulfide bonds within the secondary structure of proteins causes ROS accumulation (Higa and Chevet, 2012). During ER stress when protein folding is inefficient, additional rounds of folding are required to fold the proteins into their proper configurations; therefore, ER stress has been shown to cause the increased production of

ROS (Harding *et al.*, 2003; Cullinan and Diehl, 2006; Tavender ad Bulleid, 2010). To protect itself from the damaging effects of ROS accumulation, the cell could downregulate the other main source of ROS by suppressing the TCA cycle and oxidative phosphorylation.

In any case, while regulation of the Idh3α may provide some benefits to the cell during ER stress, it cannot be responsible for the viability effects seen with Hes1 RNAi. Therefore, the Hes1 target responsible for viability needs to be determined. To do this, we will identify other targets of this transcriptional repressor during ER stress, possibly by high-throughput sequencing of cells treated +/- ER stress and +/- Hes1.

Overall, our results describe the regulation of the transcriptional repressor Hes1 during ER stress. Our study also begins the investigation into the suppression of downstream metabolic targets of Hes1 like Idh3α. And finally, the regulation of metabolism by the UPR via Hes1 provides a possible mechanism to describe the metabolic shift in cancer cells known as the Warburg Effect.

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